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14. ABSTRACT
We have discovered that the Akt pathway modulates breast cancer cell survival in response to genotoxic agents, and discovered a new substrate of Akt, MERIT40, that is phosphorylated upon exposure of cells to chemotherapeutic drugs. We propose that this represents a major mechanism by which cells exposed to these drugs evade cell death by apoptosis and thus become resistant to the damaging effects of clinically-relevant chemotherapy agents. These findings have important ramifications for the use of chemotherapy drugs in breast cancer patients, and many also suggest that MERIT40 may be used as a clinically relevant biomarker for resistance to doxorubicin.

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INTRODUCTION

Genotoxic chemotherapy agents are used to treat breast cancer at all stages of the disease. However, the duration of response is frequently limited by chemotherapy resistance mechanisms. Therefore, resistance has a major impact on breast cancer patient survival. Despite the importance of this issue, the molecular mechanisms underlying resistance are poorly understood and strategies to combat chemotherapy resistance are lacking. The phoshoinositide 3-kinase (PI3K)/Akt pathway has emerged as a major regulator of numerous cellular phenotypes associated with breast cancer. In this project we hypothesized that a major mechanism of resistance to genotoxic chemotherapy agents is activation of the PI3K/Akt signaling cascade. We proposed that *genotoxic drugs induce the activation of Akt to initiate a signaling pathway that renders breast cancer cells resistant to chemotherapy*. This study has two specific aims. In Aim 1 we propose to determine specific contributions of the PI3K/Akt pathway in mediating resistance to chemotherapy drugs. In Aim 2 we propose to identify Akt substrates that mediate the response of breast cancer cells to genotoxic chemotherapy agents. Defining the contribution of the PI3K/Akt pathway to chemotherapy resistance is of great importance as a significant proportion of breast cancer patients harbor mutations in this critical signaling pathway. An understanding of the mechanisms that contribute to chemotherapy resistance will permit the development of novel strategies to treat breast cancer.

BODY

Aim 1: Determine specific contributions of the PI3K/Akt pathway in mediating chemoresistance to genotoxic drugs.

Aim 1, Task 1: Analysis of the ability of genotoxic agents to induce phosphorylation of Akt in breast cell lines. We have demonstrated that genotoxic chemotherapy agents, including etoposide, doxorubicin and cisplatin, induce Akt phosphorylation in both non-tumorigenic breast and cells breast cancer cells (Figure 1A). Akt phosphorylation is triggered by sublethal concentrations of each drug suggesting that activation of the PI3K/Akt pathway could contribute to survival signaling (Figure 1B).

We consistently observed that the DNA topoisomerase II inhibitor doxorubicin is able to induce the most significant increase in Akt phosphorylation in breast cancer cells (Figure 1A). Doxorubicin is frequently used as a single agent therapy for both primary and recurrent breast cancer [1]. However, the duration of response to doxorubicin is frequently limited either by the intrinsic resistance of primary tumors to treatment, or through the emergence of chemotherapy resistance in initially responsive tumors. We therefore decided to examine in greater detail the capacity of doxorubicin to modulate PI3K/Akt pathway activity. Akt phosphorylation occurs within hours of exposure to doxorubicin and is associated with enhanced phosphorylation of the Akt substrate PRAS40 (Figure 2A,B). Akt phosphorylation is also coincident with phosphorylation of histone H2A.X, a specific marker of DNA damage (Figure 2A,B) indicating that Akt phosphorylation occurs as an early response to doxorubicin treatment and within the same time frame as DNA damage. The ability of doxorubicin to induce Akt phosphorylation is prevented by pre-treating cells with a PI3K inhibitor (BKM120) or an inhibitor of DNA-PK (Nu7441) (Figure 2C). Both PI3K and DNA-PK have previously been implicated in mediating Akt phosphorylation in response to DNA damage [2].

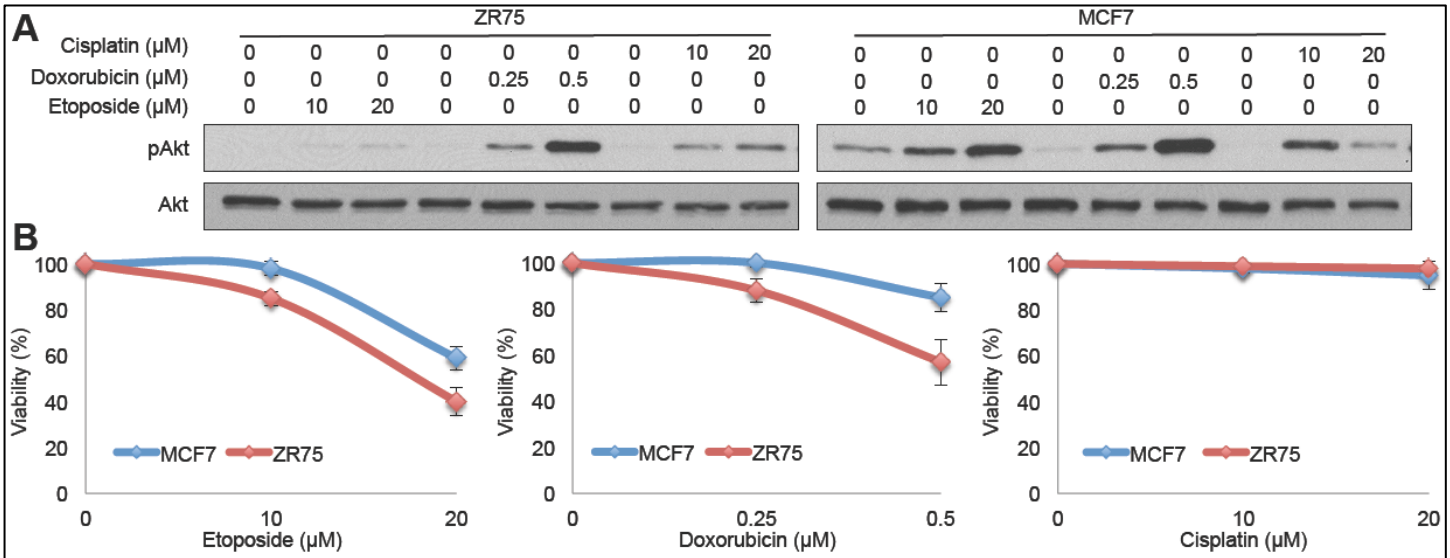

Figure 1. (A) Akt phosphorylation in ZR75 and MCF7 breast cancer cells exposed to genotoxic drugs for 24 hours. (B) Viability of ZR75 and MCF7 cells following a 48 hour exposure to genotoxic drugs.

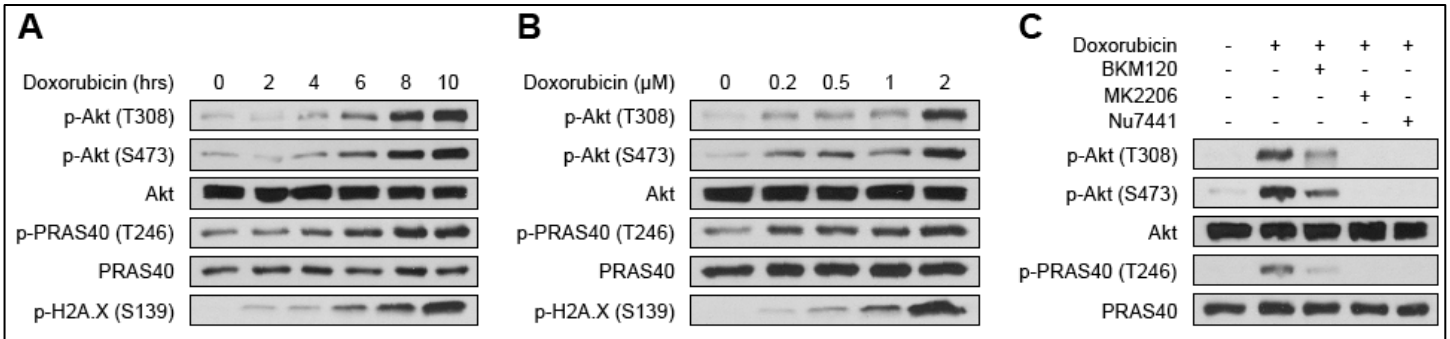

Figure 2. (A) Time-course of Akt phosphorylation in MCF10A cells exposed to doxorubicin. (B) Concentration-dependence of Akt phosphorylation in MCF10A cells exposed to doxorubicin. (C) Modulation of doxorubicin-induced Akt phosphorylation by PI3K (BKM120), Akt (MK2206) and DNA-PK (Nu7441) inhibitors.

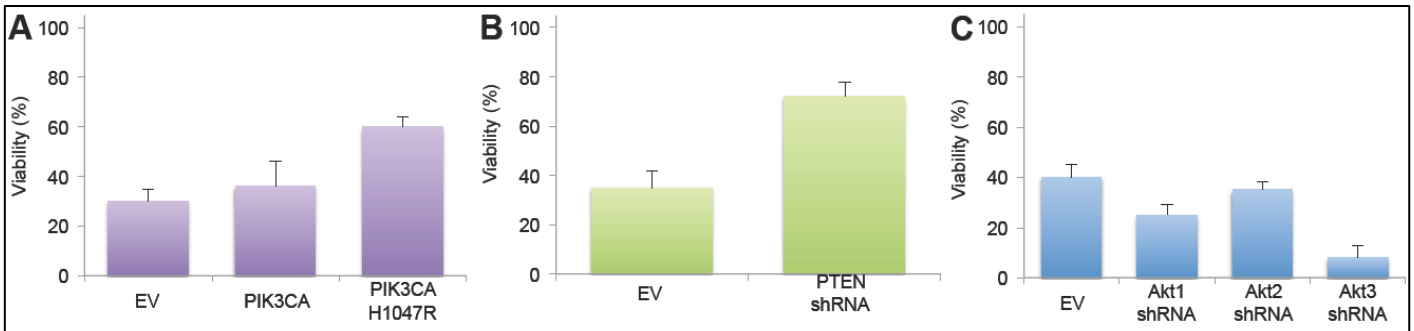

Figure 3. Viability of MCF10A cells exposed to doxorubicin following: (A) overexpression of wild-type PIK3CA or a constitutively active PIK3CA H1047R mutant, (B) shRNA-mediated silencing of PTEN or (C) shRNA-mediated silencing of Akt isoforms.

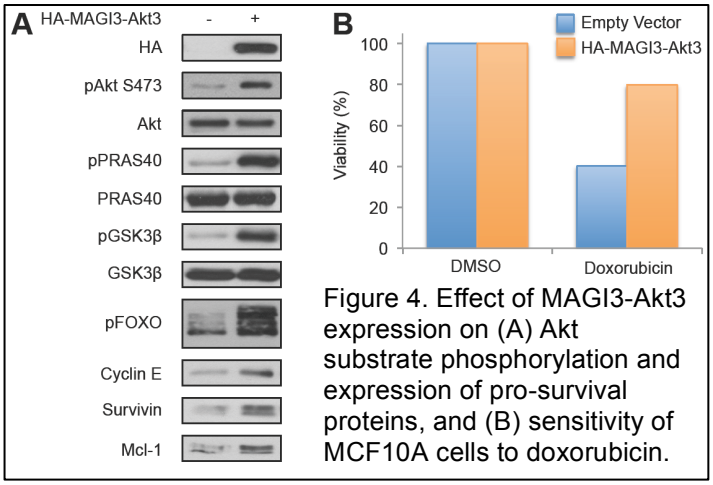

Figure 4. Effect of MAGI3-Akt3 expression on (A) Akt substrate phosphorylation and expression of pro-survival proteins, and (B) sensitivity of MCF10A cells to doxorubicin.

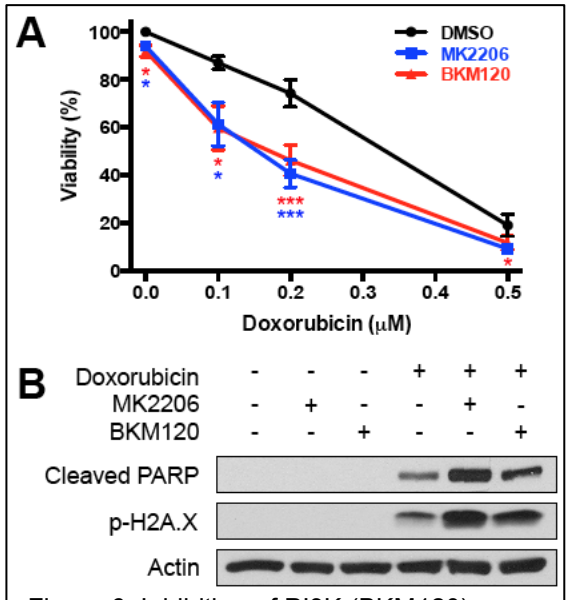

Figure 6. Inhibition of PI3K (BKM120) or Akt (MK2206) (A) sensitizes cells to doxorubicin by (B) enhancing apoptosis, as measured by PARP cleavage, and exacerbating DNA damage, as measured by phosphorylation of histone H2A.X

Aim 1, Task 2: Examine the contribution that components of the PI 3-K signaling pathway make to the cellular response to genotoxic stress. We have shown that modulation of PI3K/Akt activity alters cellular sensitivity to genotoxic agents. Hyperactivation of the PI3K pathway, induced by overexpression of the constitutively active catalytic subunit of PI3K (PIK3CA H1047R) (Figure 3A) or PTEN knockdown (Figure 3B), renders MCF10A cells more resistant to doxorubicin. In contrast, silencing of Akt isoforms sensitizes MCF10A cells to doxorubicin with Akt3 appearing to play a dominant role in regulating cellular viability (Figure 3C).

Having observed that Akt3 makes a significant contribution to doxorubicin sensitivity we decided to investigate the ability of the MAGI3-Akt3 fusion protein to confer resistance to genotoxic agents. A whole-exome and whole-genome sequencing effort to discover mutations and gene rearrangements in human breast cancers identified a translocation event between the *Akt3* gene and the *MAGI3* gene that produces an in-frame fusion protein [3]. This is the first fusion protein to be identified in the PI 3-K/Akt pathway. The translocation event results in significant disruption to the integrity of both Akt3 and MAGI3. We have demonstrated that MAGI3-Akt3 is constitutively phosphorylated in the Akt3 kinase domain in the absence of growth factors and its expression enhances Akt substrate phosphorylation [3] (Figure 4A). We have also demonstrated that MAGI3-Akt3 elevates the expression of pro-survival proteins including survivin and Mcl-1 (Figure 4A) and renders MCF10A cells more resistant to doxorubicin (Figure 4B). This suggests that Akt3 signaling makes a significant contribution to chemotherapy resistance. Using a combination of knockdown and overexpression studies we are continuing to examine the mechanisms by which Akt3 can influence the response of breast cancer cells to genotoxic drugs.

Aim 1, Task 3: Examine combination therapy with DNA damaging agents and chemical inhibitors of the PI3K/Akt pathway as an approach to kill breast cancer cells. We have observed activation of PI3K/Akt signaling as an early response to genotoxic drug exposure (Figure 2A). The PI3K/Akt pathway is known to contribute to cellular survival by

enhancing cell proliferation and blocking apoptosis. We therefore hypothesized that inhibition of PI3K/Akt signaling might sensitize breast cancer cells to genotoxic chemotherapy agents by eliminating a major survival pathway. Indeed, pretreatment with a PI3K inhibitor (BKM120) or an Akt inhibitor (MK2206) rendered MCF10A cells markedly more sensitive to the cytotoxicity of doxorubicin (Figure 6A). Combination treatment with PI3K/Akt pathway inhibitors and doxorubicin was associated with an increase in the abundance of cleaved PARP, a specific marker of apoptosis (Figure 6B). In addition, disruption of PI3K/Akt signaling enhanced doxorubicin-induced DNA damage, as measured by an increase in phosphorylation of H2A.X (Figure 6B). These results indicate that PI3K/Akt signaling contributes to the resolution of DNA damage and maintenance of cell viability following exposure to genotoxic drugs. The ability of PI3K/Akt inhibitors to sensitize to doxorubicin was also observed in T47D, SUM159 and MCF7 breast cancer cells (Figure 7). This data suggests that

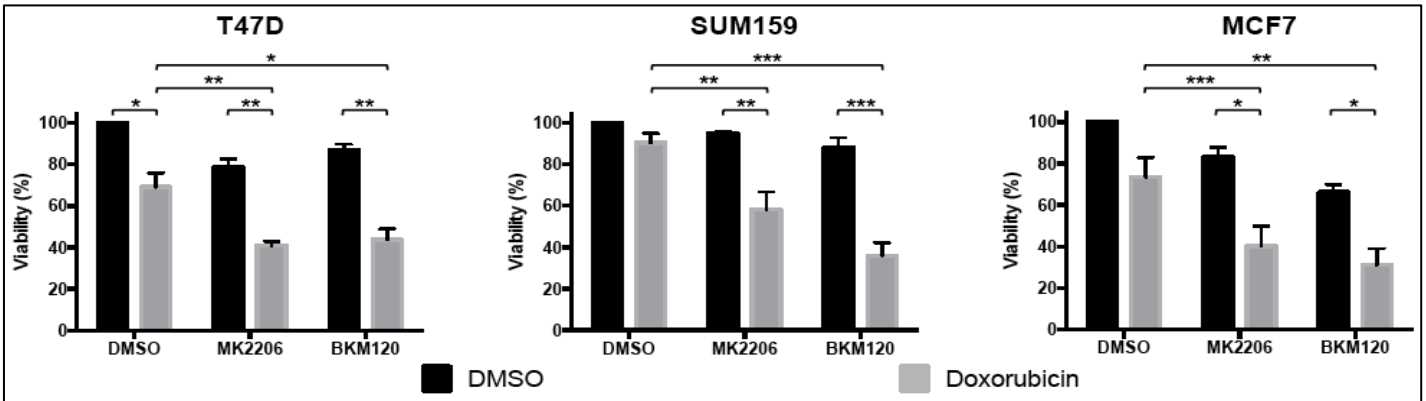

Figure 7. Inhibitors of PI3K (BKM120) or Akt (MK2206) sensitize T47D, SUM159 and MCF7 cells to doxorubicin

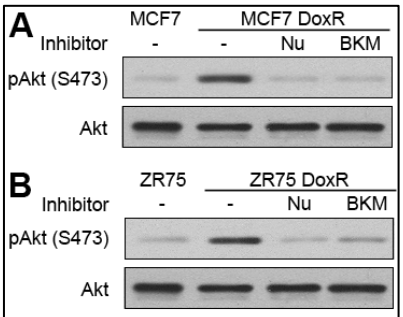

Figure 8. Doxorubicin-resistant (DoxR) (A) MCF7 and (B) ZR75 cells exhibit higher Akt phosphorylation which is abrogated by DNA-PK or PI3K inhibition.

combining PI3K/Akt inhbitors and genotoxic drugs could be a beneficial strategy for breast cancer therapy.

To examine the ability of PI3K/Akt inhibitors to overcome acquired resistance to doxorubicin we have established chemotherapy resistant breast cancer cell lines. The resistant lines were generated by chronic exposure to low dose doxorubicin over a six month period. Basal Akt phosphorylation is significantly higher in the resistant lines (Figure 8). We observed that the enhanced Akt activity in doxorubicin-resistant cells was sensitive to both DNA-PK and PI3K inhibition, suggesting that prolonged exposure to genotoxic agents induces feedback activation of PI3K signaling (Figure 8). We are continuing to investigate the role of feedback activation of the PI 3-K pathway in mediating acquired chemotherapy resistance. We will further explore the idea that downregulation of PI 3-K pathway activity, with shRNA or inhibitors, could resensitize resistant cell lines to chemotherapy agents.

Aim 1, Task 4: In vivo *assays to determine Akt signaling specificity.* We have developed cell lines in which we can reproducibly silence Akt isoform expression by addition of doxycycline. This system will be discussed in Aim 2. These cell lines will be used to perform xenograft experiments to examine the contribution of Akt1, Akt2 and Akt3 to the antitumor activity of doxorubicin. Based on our *in vitro* studies (Figure 3C) we expect to show that Akt isoform depletion enhances the anti-tumor efficacy of doxorubicin. We will pay particular attention to any differences in the ability of individual Akt isoforms to modulate the *in vivo* efficacy of doxorubicin.

Based on our finding that the MAGI3-Akt3 fusion protein promotes resistance to doxorubicin *in vitro* (Figure 4) we have

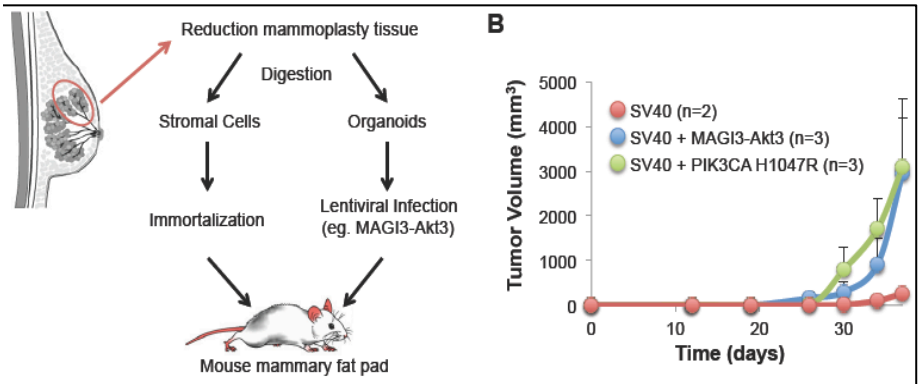

Figure 9. (A) The human-in-mouse (HIM) protocol. (B) MAGI3-Akt3 and PIK3CA H1047R induce mammary tumor formation in the HIM model.

initiated collaborative studies with Dr. Charlotte Kuperwasser (Tufts University School of Medicine, Boston, MA) to examine the oncogenic potential of MAGI3-Akt3 *in vivo*. Dr. Kuperwasser's lab has pioneered a human-in-mouse (HIM) breast xenograft model (Figure 9A). Our pilot study shows that MAGI3-Akt3 promotes mammary tumor development *in vivo*, in a manner similar to that observed when cells are transformed with the oncogenic PIK3CA H1047R mutant (Figure 9B). We are continuing to collaborate with Dr. Kuperwasser to examine the oncogenic potential of the MAGI3-Akt3 fusion protein *in vivo* and to examine the ability of MAGI3-Akt3 to modulate chemotherapy responses *in vivo*.

Aim 2: Identify isoform-specific substrates that mediate the response of breast cancer cells to DNA damaging chemotherapy.

Cell line	PI 3-K pathway mutation	Akt1	Akt2	Akt3
MDA-MB-231	KRas (G13D)	+	+	+
MDA-MB-468	PTEN mutant	+	+	+
ZR-75-1	PTEN missense	+	+	-
T47D	PIK3CA (H1047R)	+	+	-
MCF7	PIK3CA (E545K)	+	+	-
MCF10A	-	+	+	+

Table 1. Breast cell lines utilized for inducible knockdown of Akt isoforms.

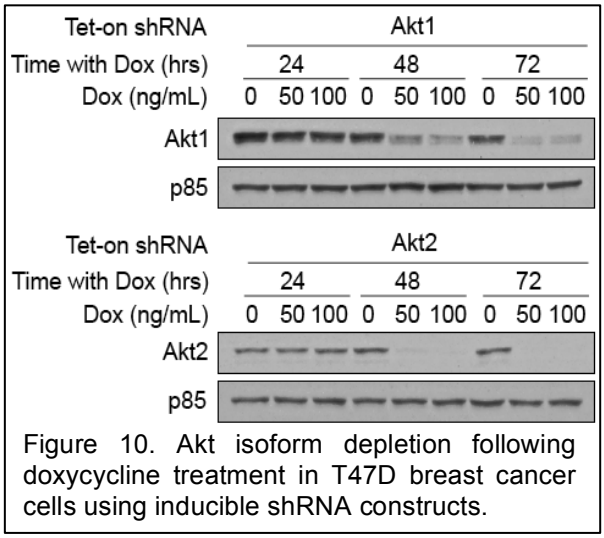

Figure 10. Akt isoform depletion following doxycycline treatment in T47D breast cancer cells using inducible shRNA constructs.

Aim 2, Task 1: Silencing Akt isoforms in breast cell lines. We have developed highly specific inducible (Tet-on) shRNA constructs to silence Akt1, Akt2 and Akt3. These constructs have been introduced into a variety of breast cancer cell lines and non-tumorigenic MCF10A breast epithelial cells (Table 1). The breast cancer cell lines have been chosen as they are known to contain hyperactivating mutations in the PI 3-K pathway. We have generated stable cell lines and confirmed that doxycycline treatment can specifically silence Akt isoforms (Figure 10). We will utilize the inducible-shRNA system to identify potential Akt isoform-specific substrates.

Aim 2, Task 2: Identify Akt substrates downstream of exposure to genotoxic agents. A major goal of this task was to evaluate MERIT40 as a novel Akt substrate contributing to the cellular response to genotoxic chemotherapy treatment. MERIT40 is a component of the nuclear BRCA1 A complex which contains Abraxas, Rap80, BRCC36, BRE and the tumor suppressor protein BRCA1 [4-6]. MERIT40 also participates in a cytoplasmic complex with ABRO1, BRCC36 and BRE [7]. Phospho-proteomic studies have demonstrated phosphorylation of MERIT40 at Ser29 in a consensus sequence that conforms to the Akt phosphorylation motif (RxRxxS/T). Using a combination of biochemical and molecular genetic approaches, we have identified MERIT40 as a novel Akt substrate. We have collaborated with Cell Signaling Technology to develop an antibody that specifically recognizes MERIT40 when phosphorylated at Ser29. Using a combination of site-directed mutagenesis and shRNA approaches we have confirmed that the phospho-MERIT40 antibody specifically recognizes MERIT40 when phosphorylated at Ser29 (Figure 11). We have also developed an antibody that recognizes total MERIT40 protein.

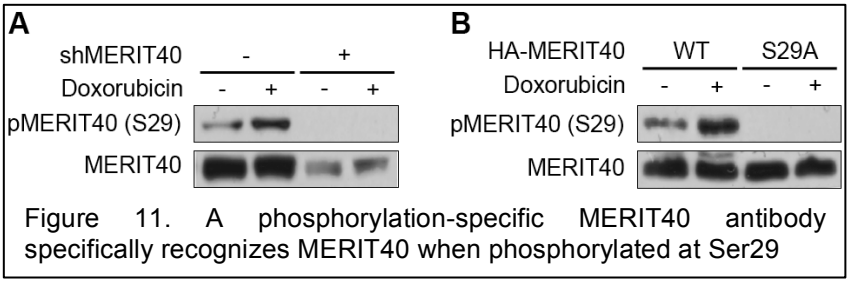

Figure 11. A phosphorylation-specific MERIT40 antibody specifically recognizes MERIT40 when phosphorylated at Ser29

Aim 2, Task 3: Validate candidates as substrates of the PI 3-K pathway in breast cancer cells. We have utilized the phospho-MERIT40 and total MERIT40 antibodies to validate MERIT40 as a novel Akt substrate. Hyperactivation of the PI3K pathway, resulting from introduction of constitutively active catalytic subunits of PI3K (PIK3CA H1047R or PIK3CA E545K) into MCF10A cells, enhances MERIT40 phosphorylation and phosphorylation of known Akt substrates like PRAS40 (Figure 12A). A similar response is observed when Akt signaling is hyperactivated by introducing constitutively active myristoylated Akt isoforms into MCF10A cells (Figure 12B). We also observe an increase in MERIT40 phosphorylation in cells exposed to doxorubicin (Figure 12C). Phosphorylation of MERIT40, induced upon doxorubicin treatment, is coincident with Akt

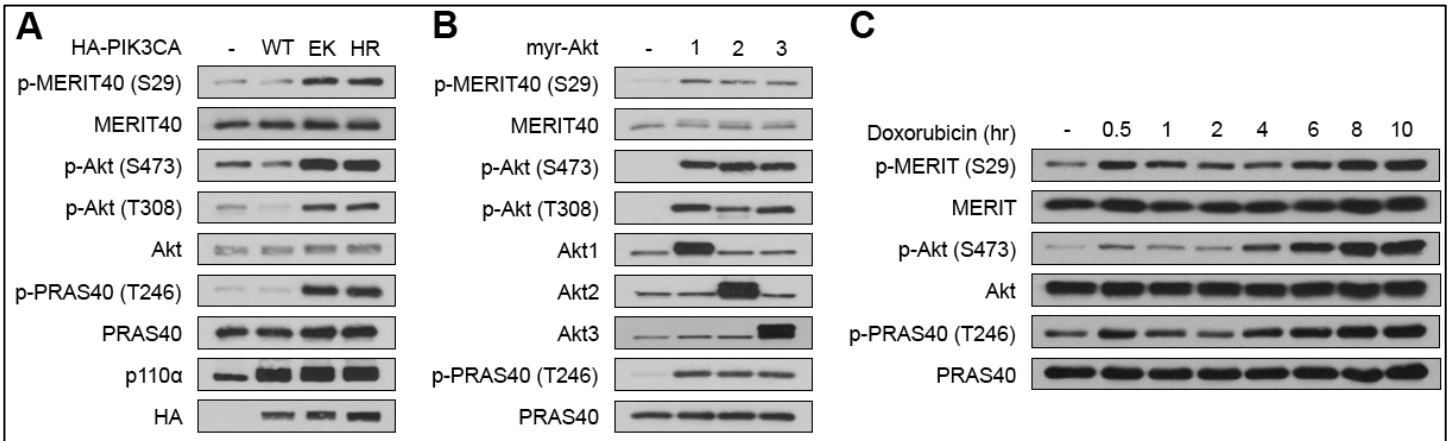

Figure 12. Hyperactive (A) PI3K or (B) Akt signaling drives MERIT40 phosphorylation. (C) Doxorubicin induces phosphorylation of MERIT40

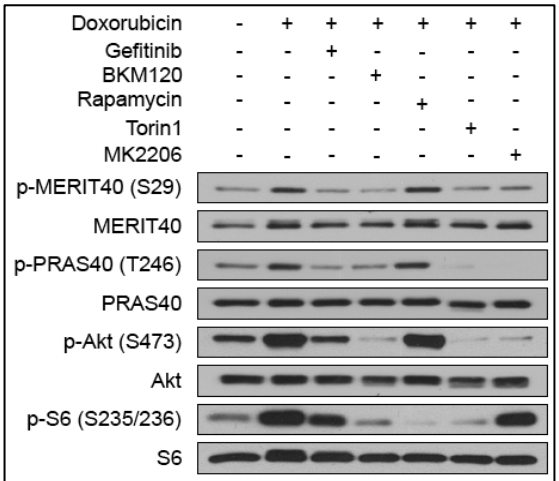

Figure 13. Modulation of doxorubicin-induced MERIT40 phosphorylation by PI3K/Akt pathway inhibitors.

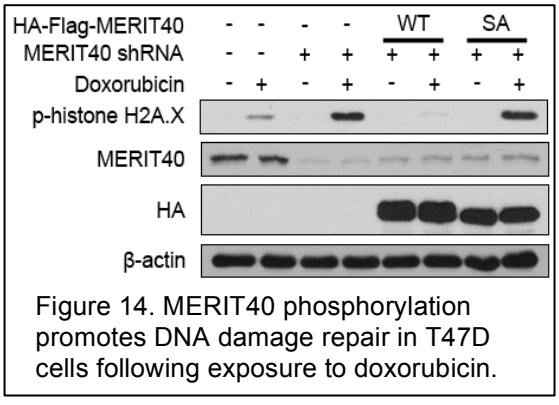

Figure 14. MERIT40 phosphorylation promotes DNA damage repair in T47D cells following exposure to doxorubicin.

phosphorylation and phosphorylation of the Akt substrate PRAS40. The ability of doxorubicin to induce MERIT40 phosphorylation is disrupted when cells are pretreated with PI3K/Akt pathway inhibitors including the PI3K inhibitor (BKM120) and the Akt inhibitor (MK2206) (Figure 13).

Aim 2, Task 4: Explore the functional consequences of candidate substrate phosphorylation by Akt in the response of breast cancer cells to DNA damaging chemotherapy. Having characterized MERIT40 as a *bona fide* Akt substrate we sought to determine the contribution of phosphorylation in regulating the cellular functions of MERIT40. As a component of the nuclear BRCA1 A complex MERIT40 contributes to DNA damage repair. We observed that MERIT40 depletion enhances DNA damage in breast cancer cell lines exposed to doxorubicin (Figure 14). Importantly, wild-type MERIT40, but not phospho-deficient MERIT40 Ser29Ala, rescues this response. This data suggests that MERIT40 phosphorylation is required to promote DNA damage repair in cells exposed to genotoxic agents. We have further demonstrated that MERIT40 phosphorylation is required for stabilization of the BRCA1 A complex (Figure 15). Doxorubicin promotes the association of endogenous MERIT40 with Rap80, another component of the BRCA1 A complex (Figure 15A). This association is sensitive to the Akt inhibitor MK2206. We have also shown that a Ser29Ala MERIT40 mutant is unable to interact with Rap80 (Figure 15B). Therefore, disruption of MERIT40 phosphorylation likely contributes to the ability of PI3K/Akt inhibitors to sensitize breast cancer cells to doxorubicin by disrupting the BRCA1 A complex which is required for DNA damage repair and cell survival in the face of DNA damage.

We have also observed that MERIT40 knockdown significantly inhibits the ability of doxorubicin to induce Akt phosphorylation in breast cancer cells (Figure 16A). In addition, overexpression of wild-type MERIT40, but not a MERIT40 Ser29Ala mutant enhances doxorubicin-induced Akt phosphorylation (Figure 16B). This suggests that phosphorylated MERIT40 participates in the regulation of Akt activity in response to genotoxic drug exposure. We are

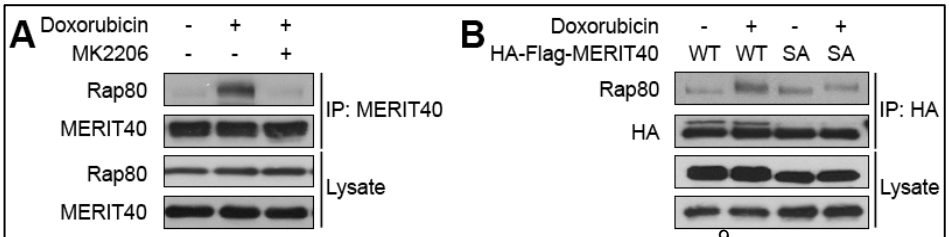

Figure 15. MERIT40 phosphorylation promotes the association of MERIT40 with Rap80.

continuing to investigate this intriguing possibility.

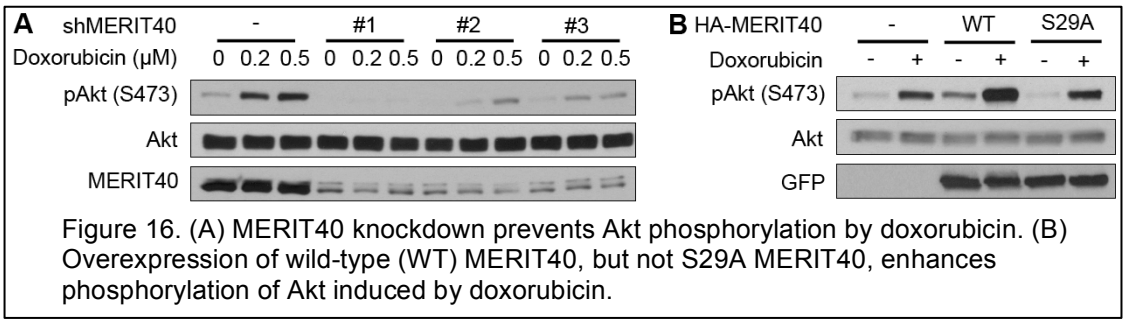

Figure 16. (A) MERIT40 knockdown prevents Akt phosphorylation by doxorubicin. (B) Overexpression of wild-type (WT) MERIT40, but not S29A MERIT40, enhances phosphorylation of Akt induced by doxorubicin.

MERIT40 has been shown to interact with the poly-(ADP-ribose) polymerase Tankyrase (TNKS) [8]. We have shown that mutation of Ser29 also disrupts the interaction between MERIT40 and TNKS (Figure 17A). Furthermore, knockdown of TNKS prevents doxorubicin-induced Akt phosphorylation in a manner analogous to knockdown of MERIT40 (Figure 16A versus 17B). TNKS has been shown to regulate pro-survival signaling pathways [9, 10] and we are currently exploring the possibility that the phosphorylation-dependent interaction between MERIT40 and TNKS protects cells from the cytotoxicity of genotoxic chemotherapy agents via modulation of Akt activity. Together, our results suggest that the Akt-dependent phosphorylation of MERIT40 induced upon doxorubicin exposure significantly contributes to the cellular response to DNA damage via multiple mechansims.

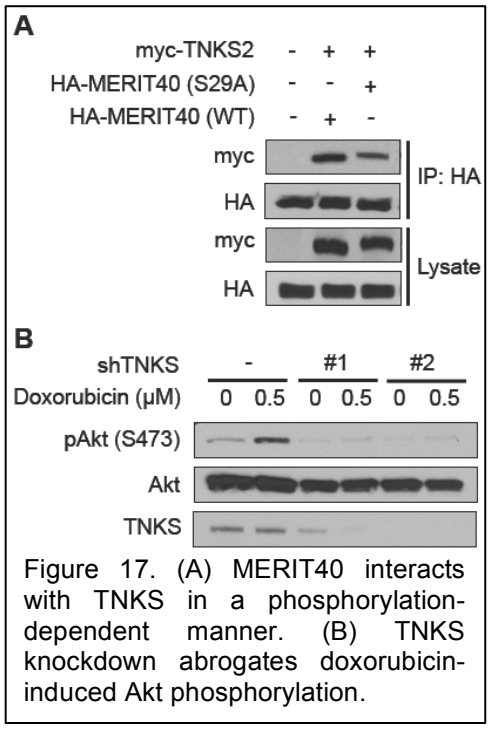

Figure 17. (A) MERIT40 interacts with TNKS in a phosphorylation-dependent manner. (B) TNKS knockdown abrogates doxorubicin-induced Akt phosphorylation.

In addition to investigating MERIT40 as a novel Akt substrate we are currently in the process of initiating phosphoproteomic studies to identify additional novel substrates involved in the response to DNA damage.

Aim 2, Task 5: Examine candidate substrate phosphorylation in human breast tissue (non-tumor and tumor) microarrays. Having identified and characterized MERIT40 as a novel Akt substrate that contributes to the cellular response to genotoxic drugs, we sought to examine the expression of MERIT40 and phospho-MERIT40 in human breast cancer specimens using human breast tissue microarrays and immunohistochemistry. All tumor and normal breast tissue samples showed staining for total MERIT40 (Figure 18A). In contrast, phospho-MERIT40 was only detected in breast tumor samples, specifically in two cases of Invasive Ductal Cancer and two cancers of Apocrine Cancer. We are currently expanding our tissue array studies to further explore the possibility that phospho-MERIT40 could be utilized as a biomarker to identify patients that might benefit from combination therapy with PI 3-K/Akt inhibitors and genotoxic chemotherapy.

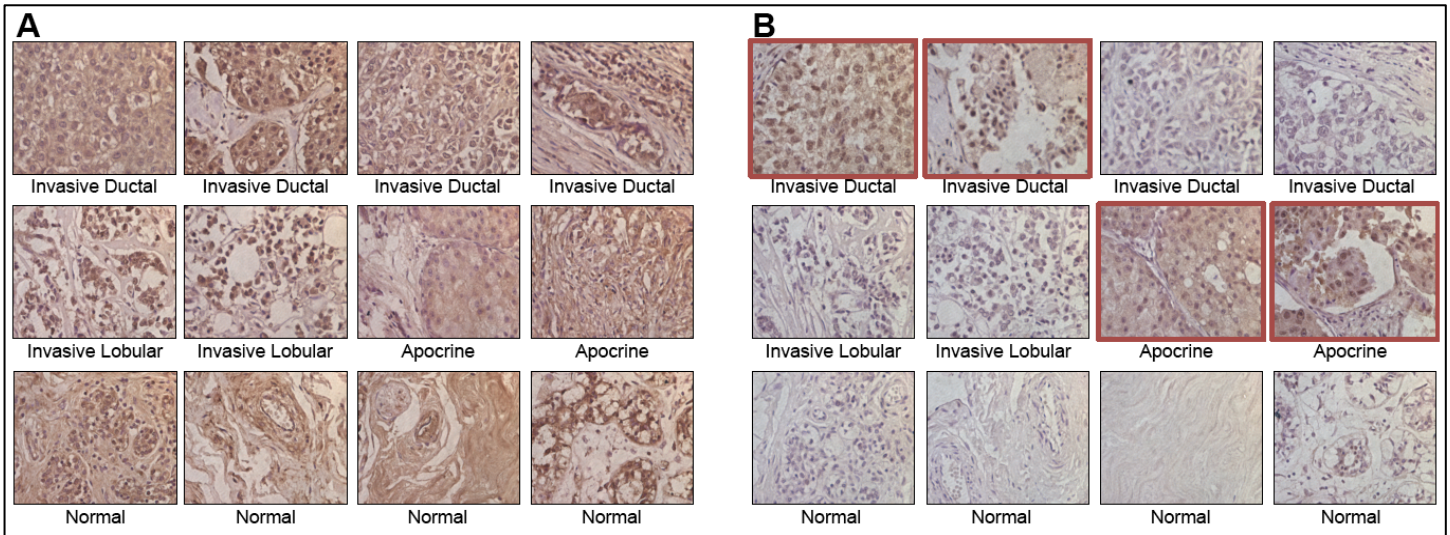

Figure 18. IHC staining of (A) MERIT40 and (B) phospho-MERIT40 in breast tissue microarrays.

KEY RESEARCH ACCOMPLISHMENTS

We have demonstrated that:

1. genotoxic chemotherapy agents induce Akt phosphorylation.
2. hyperactivation of the PI 3-K pathway renders cells more resistant to genotoxic chemotherapy drugs.
3. silencing of Akt isoforms sensitizes cells to genotoxic chemotherapy drugs. In particular, Akt3 may play a dominant role in regulating cellular sensitivity to these drugs.
4. the MAGI3-Akt3 fusion protein renders cells more resistant to genotoxic drugs.
5. inhibition of PI3K/Akt signaling enhances DNA damage and induction of apoptosis following exposure to genotoxic drugs suggesting a novel combination therapy strategy for breast cancer.
6. chemotherapy-resistant breast cancer cells exhibit enhanced basal Akt phosphorylation suggesting that prolonged exposure to genotoxic drugs induces feedback activation of PI 3-K/Akt signaling.
7. we have developed highly specific inducible shRNA constructs to silence Akt1, Akt2 and Akt3.
8. we have developed an antibody that specifically recognizes MERIT40 when phosphorylated at Ser29 by Akt.
9. MERIT40 phosphorylation is induced when cells are exposed to genotoxic drugs.
10. MERIT40 phosphorylation regulates the interaction of MERIT40 with additional components of the BRCA1 A complex, including Rap80.
11. MERIT40 phosphorylation contributes to the resolution of DNA damage following genotoxic drug exposure.
12. knockdown of MERIT40 abrogates Akt phosphorylation induced by genotoxic drugs, suggesting that MERIT40 contributes to the regulation of Akt.
13. MERIT40 interacts with tankyrase in a phosphorylation-dependent manner.
14. MERIT40 phosphorylation can be detected in human breast tumors but not in normal breast tissue.

REPORTABLE OUTCOMES

The research findings in this report and supported by this grant have been presented at the following symposia

1. Alex Toker and Kristin Brown, Keystone Symposia on PI 3-Kinase and Interplay with Other Signaling Pathways in Keystone, Colorado, February 24-28, 2013.
   Dr. Kristin Brown was awarded a Keystone Symposia Future of Science Fund scholarship based on her presentation of the work outline above.

2. Alex Toker and Kristin Brown, 2013 Annual AACR (American Association for Cancer Research) Meeting in Washington, DC, April 6-10, 2013.
   Dr. Kristin Brown was awarded a 2013 Women in Cancer Research Scholar Award.

3. A manuscript describing the identification of MERIT40 as a novel Akt substrate involved in the response to genotoxic drug exposure has been submitted for publication.

CONCLUSION

We have discovered that the Akt pathway modulates breast cancer cell survival in response to genotoxic agents, and discovered a new substrate of Akt, MERIT40, that is phosphorylated upon exposure of cells to chemotherapeutic drugs. We propose that this represents a major mechanism by which cells exposed to these drugs evade cell death and thus become resistant to the damaging effects of clinically-relevant chemotherapy agents. These findings have important ramifications for the use of chemotherapy drugs in breast cancer patients, and also suggest that MERIT40 may be used as a clinically relevant biomarker of resistance to doxorubicin.

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